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Phosphorylation of HPr by the Bifunctional HPr Kinase/P-Ser-HPr Phosphatase from *Lactobacillus casei* Controls Catabolite Repression and Inducer Exclusion but Not Inducer Expulsion

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We have cloned and sequenced the *Lactobacillus casei hprK* gene encoding the bifunctional enzyme HPr kinase/P-Ser-HPr phosphatase (HprK/P). Purified recombinant *L. casei* HprK/P catalyzes the ATP-dependent phosphorylation of HPr, a phosphocarrier protein of the phosphoenolpyruvate:carbohydrate phosphotransferase system at the regulatory Ser-46 as well as the dephosphorylation of seryl-phosphorylated HPr (P-Ser-HPr). The two opposing activities of HprK/P were regulated by fructose-1,6-bisphosphate, which stimulated HPr phosphorylation, and by inorganic phosphate, which stimulated the P-Ser-HPr phosphatase activity. A mutant producing truncated HprK/P was found to be devoid of both HPr kinase and P-Ser-HPr phosphatase activities. When *hprK* was inactivated, carbon catabolite repression of *N*-acetylglucosaminidase disappeared, and the lag phase observed during diauxic growth of the wild-type strain on media containing glucose plus either lactose or maltose was strongly diminished. In addition, inducer exclusion exerted by the presence of glucose on maltose transport in the wild-type strain was abolished in the *hprK* mutant. However, inducer expulsion of *methyl* β-D-thiogalactoside triggered by rapidly metabolizable carbon sources was still operative in *ptsH* mutants altered at Ser-46 of HPr and the *hprK* mutant, suggesting that, in contrast to the model proposed for inducer expulsion in gram-positive bacteria, P-Ser-HPr might not be involved in this regulatory process.

Bacteria can respond to the presence of abundant carbon sources via a complex network of regulatory processes. Rapidly metabolizable carbohydrates such as glucose inhibit expression of catabolic operons encoding the enzymes necessary for the uptake and metabolism of less-favorable carbon sources (43) and also affect the synthesis of enzymes of central metabolic pathways such as glycolytic enzymes, as has been shown in *Lactobacillus casei* (31) and *Lactococcus lactis* (28). In gram-positive bacteria, the metabolite-activated HPr kinase/P-Ser-HPr phosphatase (HprK/P) (2, 13, 22, 38) is the first enzyme of the signal transduction pathway used to control several of these regulatory processes. This bifunctional enzyme catalyzes the phosphorylation of HPr at the regulatory seryl residue 46 (5) as well as the dephosphorylation of seryl-phosphorylated HPr (P-Ser-HPr) (22). HPr is a phosphocarrier protein of the phosphoenolpyruvate (PEP):carbohydrate phosphotransferase system (PTS). The PTS catalyzes the concomitant uptake and phosphorylation of carbohydrates by forming a protein phosphorylation cascade. HPr becomes intermediately phosphorylated by PEP and enzyme I at the catalytic His-15, and P-His-HPr transfers the phosphoryl group to the sugar-specific enzyme II complexes which transport and phosphorylate their corresponding substrates (35).

Carbon catabolite repression (CCR) in gram-positive bacteria was found to be one of the P-Ser-HPr-controlled processes. In *Bacillus subtilis* and *L. casei*, the activity of several catabolic enzymes was partly or completely relieved from repression by glucose or other rapidly metabolizable carbon sources when Ser-46 of HPr was replaced with an alanine (*ptsH1* mutation) (6, 11, 29, 51, 59). P-Ser-HPr was found to bind with high affinity to the catabolite control protein A (CcpA) (4, 20), a member of the LacI/GalR family of transcriptional repressors/activators (17). Binding of P-Ser-HPr allows CcpA to interact with the *cis*-acting catabolite response elements (*cre*) (10, 11, 14, 21, 29, 36), which are located in front of or within the 5′ region of many operons (18, 33), leading either to reduced expression of catabolite-repressed genes (CCR) or to enhanced expression of catabolite-activated genes (carbon catabolite activation [CCA]) (36, 49).

B. subtilis was found to possess an HPr-like protein, Crh (catabolite repression HPr), in which the catalytic His-15 was replaced with a glutamine, but which contained Ser-46 (12). As a consequence, Crh was not phosphorylated with PEP and enzyme I. By contrast, HprK/P was able to catalyze the ATP-dependent phosphorylation of Crh at Ser-46 (12, 30). Similar to P-Ser-HPr, P-Ser-Crh is implicated in CCR and CCA. In a *ptsH1* mutant, several genes and operons were not or only partly relieved from CCR or CCA. The residual CCR and CCA observed in *ptsH1* mutants disappeared almost completely when *crh* was disrupted or when the *crh* gene was exchanged for the *crh1* allele (replacement of Ser-46 of Crh with an Ala) (11, 12, 29, 36, 49). Similar to P-Ser-HPr, P-Ser-Crh seems to act as corepressor for CcpA, allowing binding of the repressor to several *cre* sites (11, 29, 36).

In addition to its participation in CCR, P-Ser-HPr has been suggested to inhibit the glucose- and lactose-specific non-PTS permeases of *Lactobacillus brevis* by an inducer exclusion mechanism (56, 57) and to stimulate sugar-P phosphatases

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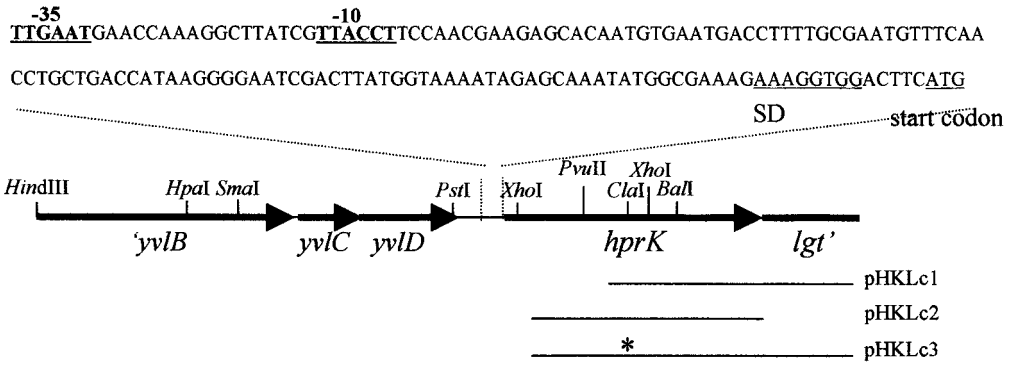

FIG. 1. Schematic presentation of the 3,139-bp-long cloned and sequenced chromosomal *L. casei* DNA fragment containing the *hprK* gene. Indicated are the five ORFs (*yvlB*, *yvlC*, *yvlD*, *hprK*, and *lgt*) detected in this fragment and several restriction sites. The DNA sequence shown above this scheme represents the region preceding *hprK* and includes a putative promoter (−10 and −35), a presumed ribosome binding site (SD), and the ATG start codon. The *L. casei* DNA fragments present in the plasmids pHKLc1, pHKLc2, and pHKLc3 are aligned underneath the schematic presentation of the cloned *L. casei* DNA. The asterisk in pHKLc3 indicates the position of the *hprK208*(Am) mutation.

presumed to be implicated in inducer expulsion in streptococci, lactococci, and enterococci (54, 55, 58). During inducer expulsion, nonmetabolizable carbohydrates such as *methyl* β-D-thiogalactoside (TMG) or 2-deoxy-D-glucose, which are taken up by the PTS and accumulated in the cell as phospho derivatives, are expelled in a two-step process triggered by the presence of a rapidly metabolizable carbon source (41, 46). The accumulated phosphorylated carbohydrates are first dephosphorylated and are subsequently expelled from the cell in the unphosphorylated form (40). In contrast to the well-established role of P-Ser-HPr in CCR and CCA, its participation in inducer exclusion and inducer expulsion was based mainly on in vitro experiments with vesicles. *L. lactis* vesicles seemed to have lost most of their HPr, whereas other enzymes, such as HprK/P or enzyme I, remained in the vesicles (55). Electroporation of wild-type *B. subtilis* HPr into *L. lactis* vesicles preloaded with TMG-6-P was reported to stimulate glucose-mediated inducer expulsion of TMG, whereas Ser-46-Ala mutant HPr had no such effect (55). *B. subtilis* Ser-46-Asp mutant HPr, which structurally resembles P-Ser-HPr (53), was found to activate streptococcal, lactococcal, and enterococcal intracellular sugar-P phosphatases assumed to catalyze the first step of inducer expulsion (54, 55, 58). The substrate-stimulated binding of *B. subtilis* Ser-46-Asp mutant HPr to the glucose- and lactose-specific *L. brevis* H^+ symporters suggested that P-Ser-HPr might also play a role in inducer exclusion (56, 57). As Ser-46-Asp, but not Ser-46-Ala, *B. subtilis* mutant HPr electroporated into *L. brevis* vesicles slowed the uptake of the nonmetabolizable lactose and glucose analogs TMG and 2-deoxy-D-glucose and promoted their export from vesicles preloaded with these sugar derivatives, it has been proposed that binding of P-Ser-HPr to the lactose- or glucose-specific H^+ symporters of *L. brevis* would lead to inducer expulsion by converting the transporters into diffusion facilitators (57).

CcpA (24), the *cre* (18), HprK/P, and HPr containing the consensus phosphorylation motif (V/G)(N/D)XKS(L/I)(M/I)(G/N)(V/L) from position 42 to 50 (13) have been detected in many gram-positive organisms. It is therefore likely that the CCR and CCA mechanism established for *B. subtilis* constitutes the major CCR and CCA mechanism operative in low guanine-plus-cytosine gram-positive bacteria. To test the importance of HprK/P in CCR in gram-positive organisms other than *B. subtilis*, we cloned the *hprK* gene from *L. casei* and constructed an *hprK* mutant in which codon 208 was converted to an amber codon. Based on in vitro results, inducer exclusion and inducer expulsion were also suggested to be controlled by P-Ser-HPr. However, these two regulatory phenomena do not seem to exist in bacilli, but have been described for lactobacilli (3, 16, 56, 57). Therefore, the *hprK208*(Am) mutant allowed, for the first time, in vivo experiments aimed to determine whether phosphorylation of HPr by HprK/P is indeed involved in inducer exclusion and inducer expulsion.

MATERIALS AND METHODS

Strains, plasmids, and culture media. *L. casei* BL23 (ATCC 393 cured of plasmid pLZ15) was used in this study. The mutant strains *ccpA*::*erm* (32), *ptsH1* (Ser46Ala), and *ptsH2* (Ser46Thr) (51) were derived from this strain. Bacteria were grown under static conditions at 37°C in MRS medium (Difco Laboratories, Detroit, Mich.) or MRS fermentation medium (Scharlau S.A., Barcelona, Spain). For diauxic growth experiments, *L. casei* strains were pregrown overnight in 1 liter of MRS basal medium containing 10 g of polypeptone, 10 g of meat extract, 5 g of yeast extract (all from Difco Laboratories), 2 g of $K_2HPO_4 \cdot 3H_2O$, 5 g of sodium acetate, 2 g of dibasic ammonium citrate, 0.1 g of $MgSO_4$, 0.05 g of $MnSO_4$, 1 ml of Tween 80, and 5 g of glucose. The overnight culture was used to inoculate 30 ml of fresh basal medium containing 0.05% glucose plus either 0.05% lactose or 0.05% maltose at an optical density at 550 nm (OD_{550}) of 0.05, which was subsequently incubated at 37°C. Samples of 1 ml were withdrawn at the indicated time intervals to monitor growth by measuring the OD_{550}.

Escherichia coli NM522 (Appligene Oncor Lifescreen, Watford, United Kingdom), M15[pREP4] (QIAGEN, Chatsworth, Calif.), and ECE89 (45) were grown with shaking at 37°C in Luria-Bertani medium. Cloning experiments were carried out as previously described (44), and transformed bacteria were plated on solid media containing 1.5% agar. The antibiotic concentrations for selecting *E. coli* transformants were 100 μg of ampicillin per ml or 25 μg of kanamycin per ml and 5 μg of erythromycin per ml for the selection of *L. casei* integrants.

The plasmids used in this study were pBC KS^+ (Stratagene, La Jolla, Calif.), pQE30 (Qiagen), pUSH1 (45), and the pBluescript SK(−)-derived integrative vector for lactobacilli, pRV300 (26).

DNA amplification by PCR. PCR aimed to obtain fragments of the *L. casei* *hprK* gene were carried out with *Taq* DNA polymerase (Appligene) by using chromosomal *L. casei* DNA as a template and the following oligonucleotides: ohprKLc1 (5′-GGNRTNGGNAARAGYGARAC-3′) based on the conserved sequence GIGKSET present in most HprK/P around position 160 and ohprKLc2 (5′-RAARTTNCCCCANCGNCC-3′) based on the conserved sequence GRWGNF present in prolipoprotein diacylglyceryl transferases (Lgt) (37) or ohprKLc3 (5′-ATA*AAGCTT*GARMTGACNGGNTAYTTYRAYTWYTA-3′) based on the conserved sequence ELTGYFNYY present around position 40 in HprK/Ps and ohprKLc4 (5′-ATTGAAAA*GAGCTC*GGATTAAGTGCT-3′). ohprKLc3 and ohprKLc4 contain restriction sites for *Hin*dIII and *Sac*I, respectively, which are indicated in italics. Oligonucleotide ohprKLc4 corresponds to the sequence located 9 to 35 bp downstream from the *hprK* stop codon. The C at position 10 of ohprKLc4 was replaced with an A and the A in position 12 was replaced with a C to allow the creation of the *Sac*I site. To exclude errors introduced by PCR, each DNA fragment was amplified in at least two independent experiments, was cloned into pBC KS^+ (Stratagene) (cut with *Eco*RV or *Hin*dIII and *Sac*I), providing plasmids pHKLc1 and pHKLc2, respectively (Fig. 1), and was sequenced on a Perkin-Elmer Abiprism 373 automated se-

quencer. The fragment of the *hprK* gene in pHKLc1 was oriented in the same direction as the *lacZ* fragment.

To purify *L. casei* HprK/P carrying a His tag, PCR amplification was carried out by using chromosomal *L. casei* DNA as a template and the two oligonucleotides 5′-GTG*GGATCC*ATGGCAGACAGCG-3′ and 5′-TAC*GGTACC*AATG AACTTCCA-3′ containing a *Bam*HI and a *Kpn*I restriction site, respectively (in italics). The resulting 1,033-bp fragment containing the complete *hprK* gene was cut with *Bam*HI and *Kpn*I and was cloned into plasmid pQE30 (Qiagen) cut with the same restriction enzymes to give pQEHKLc. The correct sequence of the amplified *hprK* was confirmed by DNA sequencing.

DNA preparation and modification. Plasmid DNA was prepared from *E. coli* cells by using the Qiagen Miniprep kit (Qiagen). *L. casei* chromosomal DNA was prepared by using the Purogene DNA isolation kit (Gentra System Inc., Minneapolis, Minn.). DNA-modifying enzymes were used as recommended by their manufacturers (New England Biolabs, Beverly, Mass., or Appligene).

In vitro mutagenesis. A point mutation was introduced into *L. casei hprK* by carrying out a PCR by using plasmid pHKLc2 as a template and the two oligonucleotides ohprKLc5 (5′-CCCCTCGAG*GTCGAC*GGTATGGATAAGCTTG A-3′), which contained part of the multiple cloning site of pHKLc2 including a *Sal*I restriction site (in italics) and a replacement of the C in position 21 by a G (underlined) destroying the *Cla*I site, and ohprKLc6 (5′-CATGAC*ATCGAT*A ATGCCCTAGCCACGAATTTC-3′). ohprKLc6 was based on the DNA sequence from position 610 to 643 of *L. casei hprK* containing a *Cla*I site (in italics). In position 20 of ohprKLc6, a T is present instead of an A, changing the leucine-encoding TTG triplet 208 of *L. casei hprK* to the amber codon TAG (underlined, reverse complementary). The resulting 522-bp PCR fragment was digested with *Sal*I and *Cla*I and cloned into pHKLc1 cut with the same enzymes, thus providing pHKLc3 containing the 3′ part of *hprK* with the amber mutation and the 5′ part of *lgt* (Fig. 1). Plasmid pHKLc3 was digested with *Hin*dIII and *Sac*I, and the resulting 1,312-bp fragment was cloned into the integrative vector pRV300 (26) cut with the same enzymes to give the 4.8-kb plasmid pHKLc208(Am).

Preparation of electrocompetent cells and electroporation. Plasmid pHKLc208(Am) was transformed into *L. casei* by electroporation by using a Gene-pulser apparatus (Bio-Rad Laboratories, Richmond, Calif.) as previously described (34).

Southern-blot hybridization. Southern blot hybridization was carried out with 5 μg of chromosomal DNA from *L. casei* which was digested with 40 U of *Hin*dIII, separated by electrophoresis on an agarose gel, and transferred to a Hybond-N membrane (Amersham Corp., Arlington Heights, Ill.). Hybridization was carried out overnight at 65°C by using a 590-bp internal fragment of the *L. casei hprK* gene as a probe. This fragment was obtained from plasmid pHKLc2 by digestion with *Hin*dIII and *Xho*I. The probe was labeled by random priming with [α-^{32}P]dCTP by using the Megaprime DNA labeling kit (Amersham) according to the supplier's suggestions.

Protein purification. In order to purify His-tagged *L. casei* HprK/P, *E. coli* M15[pREP4] (Qiagen) was transformed with plasmid pQEHKLc. A resulting transformant was isolated and grown in 1 liter of Luria-Bertani medium (Difco) at 37°C until it reached an OD_{595} of about 0.7. Preparation of crude extracts and purification of His-tagged HprK/P were carried out as previously described (12). After the last purification step, HPrK/P was dialyzed against 50 mM Tris-HCl buffer, pH 7.4, containing 0.1 mM dithiothreitol and 0.1 mM phenylmethylsulfonyl fluoride and subsequently stored at −80°C.

His-tagged *B. subtilis* HPr was purified as previously described (13). *B. subtilis* P-Ser-HPr was prepared by using the *B. subtilis* HPr kinase (22). His-tagged *B. subtilis* HprK/P was overproduced and purified as previously described (13), and *B. subtilis* Ser-46-Ala mutant HPr was obtained as previously described (8). To overproduce HPr from *L. casei*, a DNA fragment carrying the *L. casei ptsH* gene with *Bam*HI and *Hin*dIII restriction sites at the 5′ and 3′ ends, respectively, was amplified by PCR by using plasmid pVMH1 as template (51). After verifying the correct sequence, the *L. casei ptsH* gene was inserted into the shuttle vector pUSH1 (45). The resulting plasmid pLCHPr was transformed into *E. coli* ECE89 for overproduction of *L. casei* HPr as previously described (45). Since the His-tagged HPr did not bind to Ni-nitrilotriacetic acid columns, the crude extract was kept for 10 min at 75°C, and precipitated proteins were removed by centrifugation. The heat-stable HPr remained in solution, allowing almost pure HPr to be obtained from *L. casei*.

HPr kinase and P-Ser-HPr phosphatase assays. In order to measure HPr kinase and P-Ser-HPr phosphatase activities in crude extracts of integrants carrying pHKLc208(Am), cells were grown in 10 ml of MRS medium, were harvested by centrifugation, and were resuspended in 800 μl of 50 mM Tris-HCl buffer, pH 7.4, before the cells were broken by sonication (Branson sonifier 250). To demonstrate HPr kinase activity in these *L. casei* crude extracts, ATP-dependent phosphorylation assays were carried out in the presence or absence of 1.5 μg of $HPr(His)_6$ of either *L. casei* or *B. subtilis* in a total volume of 20 μl containing 5 μl of crude extract, 25 μM [γ-^{32}P]ATP (0.5 μCi), 10 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.4, and 20 mM fructose-1,6-bisphosphate. The phosphorylation reaction was stopped by adding an equal volume of sample buffer (25) to the assay mixtures before loading them onto a 15% polyacrylamide gel containing 0.1% sodium dodecyl sulfate. After electrophoresis, gels were treated for 5 min with boiling 16% trichloroacetic acid before they were dried and exposed to autoradiography (Biomax MR; Kodak).

P-Ser-HPr phosphatase assays were carried out by incubating a 20-μl assay mixture containing 10 μl of crude extract, 2.5 μg of *B. subtilis* P-Ser-$HPr(His)_6$, 20 mM sodium phosphate, pH 7.2, 10 mM $MgCl_2$, and 50 mM Tris-HCl buffer, pH 7.4, for 10 min at 37°C. The dephosphorylation reaction was stopped by heating the assay mixture for 5 min at 65°C. An equal volume of sample buffer was added before HPr and P-Ser-HPr were separated on a 12.5% nondenaturing polyacrylamide gel.

To measure the effects of FBP and inorganic phosphate (P_i) on HPr kinase and P-Ser-HPr phosphatase activities, phosphorylation and dephosphorylation assays were carried out in a total volume of 20 μl containing 0.02 or 0.05 μg of purified *L. casei* $HprK/P(His)_6$, 5 mM $MgCl_2$, and 50 mM Tris-HCl buffer, pH 7.4. *B. subtilis* $HPr(His)_6$ (2.5 μg), 1 mM ATP, and varying concentrations of FBP were added for the kinase assay, whereas 2.5 μg of *B. subtilis* P-Ser-$HPr(His)_6$ and varying concentrations of sodium phosphate were included for the phosphatase assay. After incubation for 5 min at 37°C, the reactions were stopped by heating the assay mixtures for 5 min at 65°C before separating HPr and P-Ser-HPr on a 12.5% nondenaturing polyacrylamide gel.

***N*-acetylglucosaminidase assay.** Wild-type and *ccpA*, *ptsH1*, and *hprK208*(Am) mutant cells were grown in 10 ml of MRS fermentation medium to an OD_{595} of between 0.7 and 0.9, were harvested by centrifugation, and were washed twice with 10 mM sodium phosphate buffer, pH 7.2. Permeabilized *L. casei* cells were obtained as previously described (3). To measure *N*-acetylglucosaminidase activity, a 500-μl assay mixture containing 10 μl of permeabilized cells, 10 mM sodium phosphate, pH 6.8, 1 mM $MgCl_2$, and 5 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (Sigma) was incubated for 10 min at 37°C. The reaction was stopped with 500 μl of 5% Na_2CO_3, and the OD_{420} was measured.

Maltose transport and consumption. Uptake of [^{14}C]maltose and maltose consumption by resting *L. casei* wild-type and *hprK208*(Am) mutant cells were measured in the presence and absence of glucose as previously described (51).

Inducer expulsion of [^{14}C]TMG by resting cells. *L. casei* cells were grown to mid-exponential phase in 250 ml of MRS fermentation medium containing 0.5% lactose. Glucose was subsequently added to a final concentration of 0.5%, and cells were grown for a further 40 min to induce the glucose/mannose-specific PTS proteins (50). Cells were harvested by centrifugation and were washed twice with 50 mM sodium phosphate buffer, pH 7.2, containing 10 mM $MgCl_2$. Glucose and TMG uptake studies with these cells were performed as previously described (40). To measure expulsion of TMG, 2.4 mg of cells (dry weight) were resuspended in 1 ml of 50 mM Tris-maleate buffer, pH 7.2, containing 1% peptone, were prewarmed for 10 min at 37°C prior to adding [^{14}C]TMG (0.5 mM; specific activity 0.5 mCi/mmol) (Isotopchim, Ganagobie-Peyruis, France), and were incubated for an additional 10 min at 37°C. The [^{14}C]TMG-6-P-containing cells were collected by centrifugation and were rapidly resuspended in 1 ml of the above buffer kept at 37°C. Ten microliters of 0.5 M glucose or mannose was subsequently added to trigger the expulsion of TMG. Aliquots of 100 μl were withdrawn at different time intervals, rapidly filtered through 0.45-μm-pore-size filters, and washed twice with 5 ml of ice-cold 50 mM Tris-maleate buffer, pH 7.2. The radioactivity retained by the cells was determined by liquid scintillation counting.

Separation of TMG and TMG-6-P by ion exchange and thin-layer chromatography. To test whether *L. casei* accumulates TMG or TMG-6-P, cells which had taken up [^{14}C]TMG were kept for 10 min in boiling water. After centrifugation, the supernatant was loaded on a Dowex AG1-X2 column (Bio-Rad) and unphosphorylated TMG was eluted with water before TMG-6-P was eluted with 1 M LiCl (40). TMG and TMG-6-P were also separated by thin-layer chromatography on Silica gel 60 plates (Merck, Darmstadt, Germany) as previously described (1). By using these two methods, we also tested whether the radioactive galactoside taken up by *L. casei* cells was expelled as [^{14}C]TMG or [^{14}C]TMG-6-P.

Nucleotide sequence accession number. The DNA sequence of the cloned *L. casei* chromosomal DNA fragment has been submitted to the EMBL database under accession no. Y18948.

RESULTS

Cloning of a DNA fragment encoding parts of HprK and Lgt. In most low-guanine-plus-cytosine-content gram-positive bacteria, the *hprK* gene is followed by the prolipoprotein diacylglyceryl transferase-encoding *lgt* gene (13, 22, 37). *L. casei* was found to have the same gene order, since the two degenerate primers ohprKLc1 and ohprKLc2 could be used to amplify by PCR a 879-bp DNA fragment which was cloned into pBC KS^+ providing plasmid pHKLc1 and was subsequently sequenced. Analysis of the sequence data suggested that the PCR fragment encodes the 162 C-terminal amino acids of HprK/P and the 129 N-terminal amino acids of Lgt.

To obtain part of the missing sequence of the *L. casei hprK*, a PCR was carried out by using *L. casei* BL23 DNA as a template and the primers ohprKLc3 and ohprKLc4. The re-

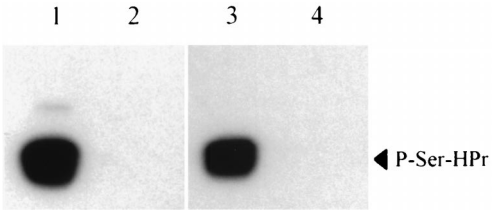

FIG. 2. [γ-^{32}P]ATP-dependent phosphorylation of *B. subtilis* and *L. casei* HPr(His)$_6$ with crude extracts prepared from either the *L. casei* wild-type strain (BL23) or the *hprK208*(Am) mutant (LcG102). Phosphorylation experiments were carried out with 1.5 μg of HPr as described in Materials and Methods. After electrophoresis, the 15% polyacrylamide gel containing 0.1% sodium dodecyl sulfate was treated with 16% boiling trichloroacetic acid, dried, and exposed to autoradiography. Lane 1, HPr from *B. subtilis* and crude extract from BL23; lane 2, HPr from *B. subtilis* and crude extract from LcG102; lane 3, HPr from *L. casei* and crude extract from BL23; lane 4, HPr from *L. casei* and crude extract from LcG102.

sulting 875-bp PCR fragment was digested with *Hin*dIII and *Sac*I and was cloned into pBC KS$^+$ cut with the same enzymes, providing plasmid pHKLc2. DNA sequencing and comparison of the translated sequence with known HprK/P sequences suggested that the amplified DNA fragment encodes amino acids 40 to 319 of *L. casei* HprK/P.

Construction of a *L. casei hprK* mutant and cloning of the entire *hprK*. In order to clone the missing 5′ part of the *hprK* gene of *L. casei* and to test whether it indeed encodes the bifunctional HPr kinase/P-Ser-HPr phosphatase, a PCR was carried out to introduce a point mutation replacing the leucine-encoding codon 208 of *hprK* with an amber codon. A 1,312-bp DNA fragment carrying the amber mutation was cloned into the integrative vector pRV300 (26), providing plasmid pHKLc208(Am) (see Materials and Methods). *L. casei* BL23 was transformed with pHKLc208(Am), and erythromycin-resistant clones were obtained. In seven clones, the integration of pHKLc208(Am) was tested by Southern blotting using as a probe a 590-bp internal *hprK* fragment as described in Materials and Methods. Only one *Hin*dIII fragment of 5.2 kb could be detected with DNA from wild-type *L. casei* BL23, whereas the seven erythromycin-resistant clones gave two bands with sizes of 3.6 and 6.5 kb (data not shown), suggesting that plasmid pHKLc208(Am), which contains a single *Hin*dIII site, had been integrated in the chromosome of these transformants. The integrants were tested for HPr kinase activity, since the Campbell-like recombination of pHKLc208(Am) with the *L. casei* chromosome can occur in two different ways giving rise to two types of integrants. Four integrants exhibited an HprK$^-$ phenotype and three exhibited an HprK$^+$ phenotype. Phosphorylation experiments with HPr(His)$_6$ from *B. subtilis* and *L. casei* and crude extracts from one of the integrants devoid of HPr kinase activity are shown in Fig. 2. Whereas phosphorylation of both HPr could be observed with crude extracts of the wild-type strain (Fig. 2, lanes 1 and 3), neither of the HPrs was phosphorylated with crude extracts of the integrant (Fig. 2, lanes 2 and 4). Sequencing of appropriate PCR products obtained from this integrant revealed that this strain, which was called LcG102, contained an *hprK* allele carrying the amber codon at position 208 and an incomplete *hprK* gene lacking the 5′ part together with the promoter region, explaining why this strain was devoid of HPr kinase activity. Sequencing of PCR products obtained with chromosomal DNA from one of the erythromycin-resistant *hprK*$^+$ strains showed that it contained a wild-type *hprK* and an incomplete *hprK* carrying the amber mutation. Our attempts to isolate from the latter strain erythromycin-sensitive *hprK* mutants resulting from a second recombination causing excision of the plasmid were not successful. The second recombination can also occur at two different sites with respect to the introduced amber codon, leading to *hprK*$^+$ or *hprK* strains. About 300 erythromycin-sensitive clones obtained after growth without selective pressure were tested and were all found to be *hprK*$^+$.

Chromosomal DNA of LcG102 was isolated, digested with *Hin*dIII, religated, and transformed into *E. coli* NM522. The plasmid present in one of the ampicillin-resistant clones was purified and found to carry an insert of approximately 3.2 kb. DNA sequencing of this plasmid (pHKLcUS) revealed that the insert contained the *L. casei* DNA fragment already present in pHKLc208(Am) and, in addition, the 5′ part of the presumed *hprK*, its promoter region, and two complete and one incomplete open reading frame (ORF) located upstream of *hprK* (Fig. 1). The proteins encoded by these three ORFs exhibited 23, 22, and 36% sequence identity, respectively, when compared to the proteins encoded by the *B. subtilis yvlB*, *yvlC*, and *yvlD* genes (23).

The presumed *L. casei hprK* gene consists of 957 bp and encodes a protein of 35,349 Da composed of 319 amino acids, which exhibits 50% sequence identity when compared to *B. subtilis* HprK/P. As in all other known HprK/P, the A motif of nucleotide binding proteins (GX_4GKS) is present around position 160. The presumed *hprK* gene starts with an ATG, which is preceded by a putative ribosome binding site located 5 bp upstream of the start codon (Fig. 1). Downstream from *hprK* and separated from *hprK* by only 1 bp, the *lgt* gene begins. The cloned *lgt* fragment encodes the first 129 amino acids of *L. casei* Lgt which exhibit 53% sequence identity when compared to the corresponding N-terminal part of *B. subtilis* Lgt.

***L. casei* HprK/P is a bifunctional enzyme regulated by FBP and P_i.** In order to confirm that the presumed *hprK* gene encodes *L. casei* HprK/P and to test whether it exhibits both HPr kinase and P-Ser-HPr phosphatase activities similar to the *B. subtilis* and *Enterococcus faecalis* enzymes (22), His-tagged *L. casei* HprK/P was purified as described in Materials and Methods. The purified enzyme phosphorylated *B. subtilis* HPr (Fig. 3A, lane 1), and this activity was stimulated by FBP (Fig. 3A, lanes 2 to 5). Phosphorylation seems to occur at Ser-46, as the *B. subtilis* Ser-46-Ala mutant HPr was not phosphorylated by the *L. casei* HprK/P (data not shown). HprK/P from *L. casei* was found to be bifunctional, as it also catalyzed the dephosphorylation of P-Ser-HPr(His)$_6$ of *B. subtilis* (Fig. 3B, lane 2). The P-Ser-HPr phosphatase activity was stimulated by P_i (Fig. 3B, lanes 3 to 5). Although the presence of P_i inhibited the ATP-dependent phosphorylation of HPr (compare Fig. 3C, lanes 1 and 2), a strong stimulation of HPr(Ser) phosphorylation by FBP occurred when the HPr kinase assays were carried out in the presence of low concentrations of P_i. When 1 mM P_i was used, almost no HPr phosphorylation could be observed in the absence of FBP, whereas in the presence of 20 mM FBP, a strong HPr kinase activity could be detected (Fig. 3C, lanes 2 and 3). By contrast, when using 8 mM P_i, FBP had almost completely lost its stimulating effect on HPr phosphorylation (Fig. 3C, lanes 8 and 9). FBP exerted only a weak inhibitory effect on P_i-stimulated P-Ser-HPr phosphatase activity (about 1.5- to twofold inhibition with 10 mM FBP, data not shown).

As already mentioned, the *hprK208*(Am) mutant LcG102 had completely lost HPr kinase activity. To test whether it was also devoid of P-Ser-HPr phosphatase activity, crude extracts of wild-type *L. casei* and the *hprK208*(Am) mutant strain were prepared, and their capacity to dephosphorylate P-Ser-HPr was assayed in the presence of 20 mM P_i. While P-Ser-HPr phosphatase activity could be clearly detected in crude extracts

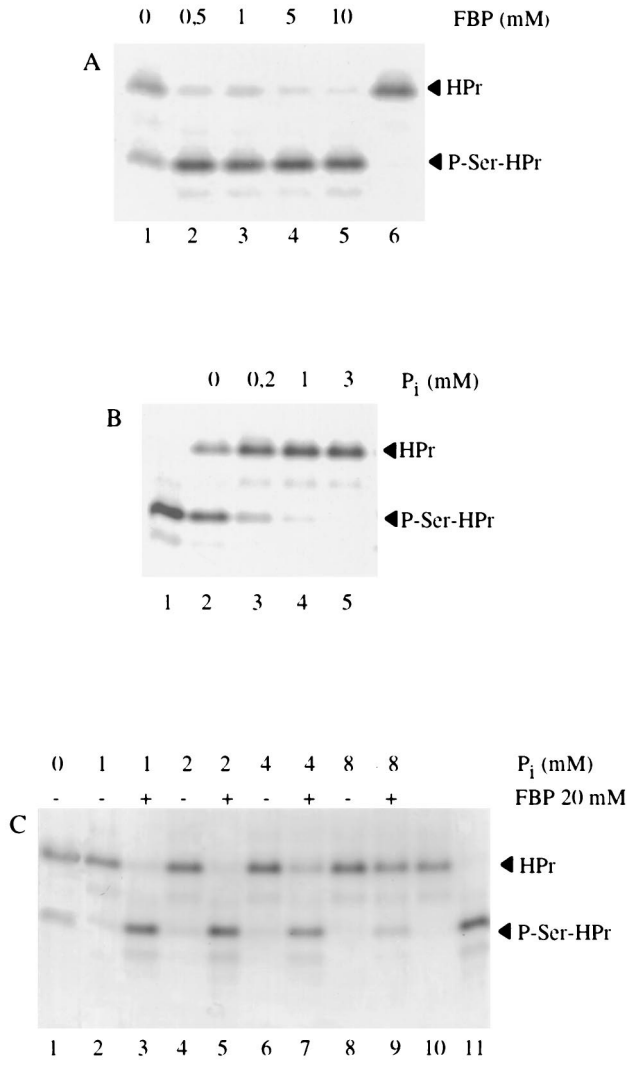

FIG. 3. The effect of FBP and P_i on ATP-dependent phosphorylation of *B. subtilis* HPr. HPr and P-Ser-HPr were separated on nondenaturing 12.5% polyacrylamide gels. (A) Effect of FBP on the *L. casei* HPr kinase activity. HPr phosphorylation was carried out with 20 ng of HprK/P and the indicated concentrations of FBP for 3 min at 37°C as described in Materials and Methods. HPr standard (2.5 μg) was loaded on lane 6. (B) Effect of P_i on *L. casei* P-Ser-HPr phosphatase activity. HprK/P-catalyzed dephosphorylation of P-Ser-HPr was performed with 50 ng of HprK/P and the indicated P_i concentrations by incubating the assay mixture for 5 min at 37°C as described in Materials and Methods. P-Ser-HPr standard (2.5 μg) was loaded on lane 1. (C) Effect of FBP on ATP-dependent HPr phosphorylation in the presence of P_i. ATP-dependent HPr phosphorylation was carried out for 5 min at 37°C with 20 ng of HprK/P and the indicated amounts of P_i in the presence (+) or absence (−) of 20 mM FBP. Lanes 10 and 11 contain 2.5 μg of HPr and P-Ser-HPr standards, respectively. After electrophoresis, gels were stained with Coomassie blue.

of the wild-type strain (Fig. 4, lanes 3 and 4), no such activity was found in crude extracts of the *hprK208*(Am) mutant LcG102 (Fig. 4, lanes 1 and 2). Even increasing the incubation time from 10 to 30 min did not allow detection of dephosphorylated HPr (data not shown).

The *hprK208*(Am) mutation affects CCR. To determine whether, similar to *B. subtilis* HprK/P (13, 38), *L. casei* HprK/P is also involved in CCR, the repressive effect of glucose on *N*-acetylglucosaminidase activity was measured in the *hprK208*(Am) mutant and compared to the activity found in the wild-type and the *ccpA* and *ptsH1* mutant strains.

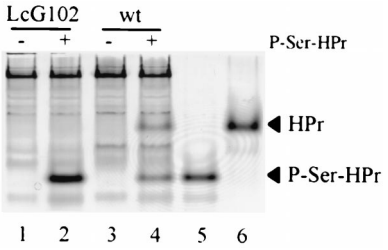

FIG. 4. P-Ser-HPr phosphatase activity in crude extracts of *L. casei* wild-type or *hprK208*(Am) mutant. P-Ser-HPr phosphatase assays were carried out with 10 μl of crude extracts in the absence (−) or presence (+) of 2.5 μg of P-Ser-HPr as described in Materials and Methods. P-Ser-HPr and HPr standards (2.5 μg each) were loaded on lanes 5 and 6, respectively. Samples were separated on a nondenaturing 12.5% polyacrylamide gel which was stained with Coomassie blue.

In wild-type strain BL23, *N*-acetylglucosaminidase activity was repressed 18-fold by the presence of glucose compared to cells grown in ribose-containing medium (Table 1). Similar to the case in *L. casei ccpA* or *ptsH1* mutants (32, 51), CCR of *N*-acetylglucosaminidase activity was strongly diminished in the *hprK208*(Am) mutant LcG102 (Table 1).

The *hprK208*(Am) mutation affects diauxic growth. Growth of the *hprK208*(Am) mutant LcG102 in MRS medium containing 0.05% glucose plus either 0.05% lactose or 0.05% maltose was compared to the growth behavior of the wild-type strain BL23. Wild-type *L. casei* grown in media containing mixtures of glucose and lactose or glucose and maltose exhibited a diauxic growth curve with two distinct growth phases separated by a lag phase of about 8 h for cells growing in glucose/lactose-containing medium and 7 h for cells growing in glucose/maltose-containing medium. In the *hprK208*(Am) mutant LcG102, the lag phase was reduced to less than 2 h for cells grown in either glucose-and-lactose- or glucose-and-maltose-containing medium (data not shown).

The *hprK208*(Am) mutation prevents the exclusion of maltose by glucose. It has recently been demonstrated that replacement of Ser-46 in *L. casei* HPr with alanine or threonine and replacement of Ile-47 with threonine prevents the exclusion of maltose by glucose (51). To test whether the effect of the *ptsH* mutations replacing Ser-46 was indeed due to the absence of ATP-dependent phosphorylation of HPr, we studied glucose-triggered maltose exclusion in the *hprK208*(Am) mutant strain LcG102. As recently reported (51), maltose uptake by wild-type cells was instantaneously arrested when glucose was added to the transport medium (Fig. 5A). By contrast, when an identical experiment was carried out with the *hprK208*(Am) mutant LcG102, maltose uptake was not inhibited but, rather, was slightly stimulated by the presence of

TABLE 1. Catabolite repression of *N*-acetylglucosaminidase in *L. casei*

L. casei strain	*N*-acetylglucosaminidase activity[a]		Repression factor
	Ribose	Glucose	
Wild-type	37.6 ± 6.7	2.0 ± 0.9	18.8
hprK208(Am)	31.5 ± 4.5	26.3 ± 1.7	1.2
ptsH1	35.3 ± 7.2	26.7 ± 6.5	1.3
ccpA	30.6 ± 4.3	19.4 ± 0.7	1.6

[a] *N*-acetylglucosaminidase activity was determined by using *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide as substrate. Activity is expressed as nmoles of *p*-nitrophenol formed per minute and per milligram of cells (dry weight). Values are given ± standard errors.

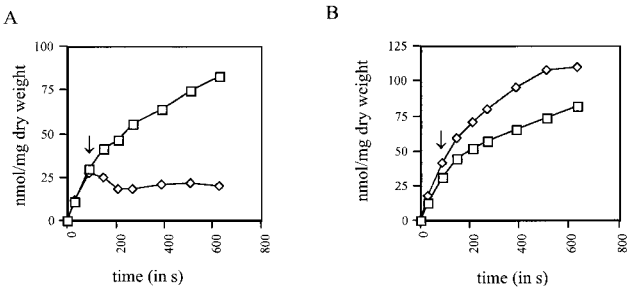

FIG. 5. [^{14}C]Maltose uptake in the presence (diamonds) and absence (squares) of glucose by *L. casei* wild-type (A) and *hprK208*(Am) mutant (B) cells. Transport studies were carried out by using the rapid filtration method. Glucose at a final concentration of 1 mM was added at the time indicated by the arrow.

glucose (Fig. 5B). The absence of glucose-triggered maltose exclusion in the *hprK208*(Am) mutant was confirmed by measuring maltose consumption in the presence and absence of 0.15% glucose with the *L. casei* wild-type and *hprK208*(Am) mutant strains. In the wild-type strain, maltose was not utilized as long as glucose was present in the growth medium (Fig. 6A), whereas maltose and glucose were simultaneously consumed by the *hprK208*(Am) mutant LcG102 (Fig. 6B).

The *hprK208*(Am) mutation does not affect inducer expulsion. For several gram-positive bacteria it has been suggested that P-Ser-HPr would be involved in inducer expulsion by activating a sugar-P phosphatase assumed to catalyze the first step of this regulatory process (54, 55, 58). By carrying out ion exchange and thin-layer chromatography, we could demonstrate that [^{14}C]TMG was accumulated by *L. casei* BL23 cells as phosphorylated derivative (more than 98%) and that after addition of glucose or mannose to these cells, the accumulated [^{14}C]TMG-6-P was rapidly dephosphorylated inside the cells before it was expelled as TMG (data not shown). When studying inducer expulsion in *ptsH* mutant strains, we observed no difference in glucose-triggered expulsion of [^{14}C]TMG measured with the wild-type BL23 and the *ptsH*S46A or *ptsH*S46T mutants (Fig. 7). The failure to detect an effect of the *ptsH* mutations on inducer expulsion could have been due to the occurrence of an *L. casei* HPr-like protein similar to Crh from *B. subtilis*. We therefore also tested inducer expulsion in the *hprK208*(Am) mutant LcG102, in which neither HPr nor an HPr-like protein can be phosphorylated with ATP. Nevertheless, glucose-triggered expulsion of [^{14}C]TMG in the *hprK208*(Am) mutant was found to be identical to that ob-

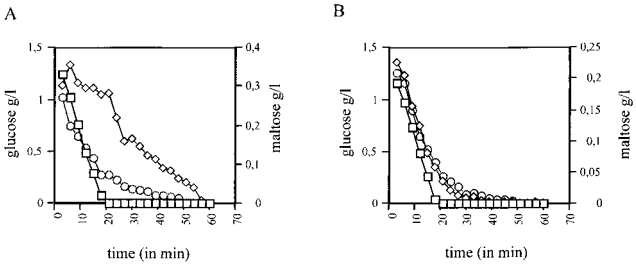

FIG. 6. Consumption of maltose (0.025%) and glucose (0.15%) by resting *L. casei* wild-type (A) or *hprK208*(Am) mutant (B) cells. The cell suspension containing 18 mg of cells (dry weight) in 5 ml of 50 mM sodium phosphate buffer, pH 7, was incubated at 37°C. Samples of 300 μl were withdrawn at the indicated time intervals and were centrifuged. The maltose concentration in experiments carried out in the presence (diamonds) or absence (circles) of glucose and the glucose concentration (squares) were determined in the supernatant.

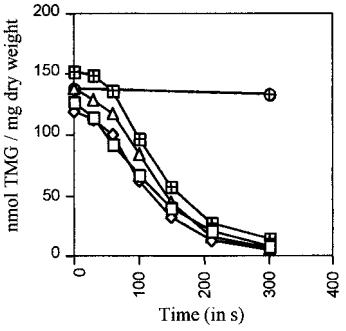

FIG. 7. Expulsion of preaccumulated [^{14}C]TMG-6-P in different *L. casei* strains. Cells preloaded with [^{14}C]TMG-6-P were washed and resuspended in 1 ml of transport buffer. At time 0, glucose was added at a final concentration of 5 mM, and 100-μl aliquots were withdrawn at the indicated time intervals. The radioactivity remaining inside the cells was measured with the wild-type strain BL23 (squares) and the *ptsH1* (triangles), the *ptsH2* (cross within squares), and the *hprK208*(Am) (diamonds) mutant strains. Two control samples were taken before glucose was added. One was immediately filtered and provided time point 0, the other was incubated at 37°C and was filtered at the end of the experiment (crosses within circles). This end point is shown only for the experiment with the *ptsH1* mutant. A similar leakage (less than 5%) of [^{14}C]TMG-6-P in the absence of glucose was observed with all other strains.

served in a wild-type strain (Fig. 7). Since P-Ser-HPr has been suggested to stimulate the sugar-P phosphatase catalyzing the first step of inducer expulsion (i.e., the intracellular dephosphorylation of accumulated sugar-P), we tested whether the galactoside was expelled as TMG or TMG-6-P from the *L. casei hprK208*(Am) mutant. Identical to the results obtained with the wild-type strain, more than 98% of the TMG expelled from the *hprK208*(Am) mutant was found to be dephosphorylated (data not shown). Very similar results were obtained when expulsion of [^{14}C]TMG was elicited with mannose instead of glucose. Mannose-triggered expulsion of [^{14}C]TMG occurred at a slightly slower rate, but again, no difference could be observed between wild-type and mutant strains (data not shown). To exclude the possibility that the *ptsH* and *hprK* mutations affected PTS transport activities, which might in an unknown way be responsible for the failure to detect a difference of TMG expulsion between wild-type and mutant strains, the uptake of [^{14}C]glucose, [^{14}C]mannose, and [^{14}C]TMG were measured and found to be identical for the wild-type strain and the *hprK208*(Am) and *ptsH* mutants (data not shown).

DISCUSSION

The bifunctional HPr kinase/P-Ser-HPr phosphatase HprK/P of gram-positive bacteria was found to be a central regulatory protein controlling the expression of catabolic (6, 11–13, 21, 29) and glycolytic (27, 31, 47) genes and probably of genes encoding enzymes implicated in the synthesis of secondary metabolites (36, 49), nitrogen metabolism (9, 59), and the Krebs cycle (47). HprK/P has also been suggested to play a role in inducer exclusion and inducer expulsion in lactobacilli and other lactic acid bacteria (3, 16, 54–58).

Here, we report cloning and sequencing of the *L. casei hprK* gene, which was found to be the first gene in an operon. The protein encoded by *hprK* was capable of phosphorylating wild-type HPr, but not Ser-46-Ala mutant HPr, and of dephosphorylating P-Ser-HPr, confirming that it is bifunctionally similar to the *B. subtilis* and *E. faecalis* HprK/Ps (22). It exhibits strong sequence identity (40 to 50%) when compared to other HPr kinases (13). The region around the glycine-rich A motif of the

nucleotide binding fold (52) is especially well conserved. HprK/Ps seem to contain an overlapping tandem repeat of the GXXXXGKS consensus sequence in the nucleotide binding site, leading to the GXXXXGKSXXGKS sequence, which is fully conserved in HprK/P of *B. subtilis* or *Mycoplasma genitalium*. In most other organisms possessing HprK/P, the first K in the above sequence was found to be replaced with a D. It is not known whether this repetition of the nucleotide binding motif is of any functional significance. In *L. casei* and all other gram-positive bacteria (except *Clostridium acetobutylicum*) for which the corresponding DNA sequence data are available, *hprK* is the first gene in an operon and always followed by the prolipoprotein diacylglyceryl transferase-encoding *lgt* gene. This conserved gene organization could be indicative of a functional interaction between HprK/P and Lgt. In the gram-negative bacteria *Neisseria gonorrhoeae*, *Neisseria meningitidis*, and *Bordetella pertussis*, *hprK* is not followed by *lgt*, but is preceded by a gene encoding an EII^{Ntr}-like protein (42), which seems to form an operon together with *hprK*. The cloned region upstream of *L. casei hprK* was found to contain one incomplete and two complete ORFs which seem to be organized in an operon. The deduced amino acid sequences exhibited similarity to proteins of unknown function encoded by the *B. subtilis yvlB*, *yvlC*, and *yvlD* genes, which are located about 11.5 kb upstream of the *B. subtilis hprK* gene (23).

The two opposing activities of recombinant purified *L. casei* HprK/P, HPr kinase and P-Ser-HPr phosphatase, were found to be regulated by FBP and P_i. HPr kinase from *L. casei* was similarly active with HPr from *L. casei* and *B. subtilis* (Fig. 2). However, in contrast to the HPr kinase activity of *B. subtilis* HprK/P, which is strongly stimulated by FBP (13, 19, 38), or of *Streptococcus salivarius* HprK/P, which is inhibited by FBP (2), the HPr kinase activity of the *L. casei* enzyme was slightly stimulated by FBP (Fig. 3A). When the experiments were carried out in the presence of 1 to 4 mM P_i, a stronger stimulatory effect of FBP on HPr phosphorylation could be observed (Fig. 3C). HprK/Ps from different organisms therefore appear to respond to similar but not identical intracellular signals, which probably reflects an adaptation to the specific physiological changes taking place in each organism when a carbon source is rapidly metabolized. P_i strongly stimulated the P-Ser-HPr phosphatase activity, as has also been observed with the *B. subtilis* and *E. faecalis* enzymes (13, 22). FBP and P_i do not seem to compete for the same binding site, as the P_i-stimulated P-Ser-HPr phosphatase activity associated with *L. casei* HprK/P was only slightly inhibited by FBP.

To study the different regulatory functions suggested for HprK/P in carbon metabolism, an *hprK* mutant has been constructed, producing a protein truncated at position 208. Crude extracts of the *hprK208*(Am) mutant strain LcG102 were not able to phosphorylate HPr from *L. casei* and *B. subtilis* (Fig. 2). They were also devoid of P-Ser-HPr phosphatase activity (Fig. 4), confirming that the major P-Ser-HPr phosphatase activity in *L. casei* is associated with HprK/P. Nevertheless, a homologue of the *B. subtilis yvoE* gene, which is located in the same operon as *hprK* (13) and which encodes an enzyme exhibiting low P-Ser-HPr phosphatase activity, is present in *Lactobacillus rhamnosus* (48), a very close relative of *L. casei*. Although the corresponding *L. rhamnosus* gene is not located in the same operon as the *hprK* gene, the purified *L. rhamnosus* YvoE homologue exhibited low P-Ser-HPr phosphatase activity similar to *B. subtilis* YvoE (V. Dossonnet and J. Deutscher, unpublished results).

Similar to *ptsH1* and *ccpA* mutants, *N*-acetylglucosaminidase activity in the *hprK208*(Am) mutant LcG102 was found to be almost completely relieved from repression by glucose (Table 1). In addition, the lag phase of about 8 h observed during diauxic growth of the *L. casei* wild-type strain in media containing glucose and either lactose or maltose was reduced to less than 2 h in the *hprK208*(Am) mutant. The fact that the lag phase had not completely disappeared is probably due to the pregrowth of cells in glucose-containing medium. When the cells were pregrown in lactose- or maltose-containing medium, the lag phase was much shorter for the wild-type strain (50) and had completely disappeared for the *hprK* mutant (data not shown). These results established that, similar to *B. subtilis*, HprK/P plays an important role in CCR in *L. casei*, and they strongly suggested that this CCR mechanism is operative in a wide variety of gram-positive bacteria, since CcpA, *cre* sites, HprK/P, and HPr with a phosphorylatable Ser-46 can be found in almost all low-guanine-plus-cytosine-content gram-positive organisms.

Results recently obtained with *L. casei ptsH* mutants, in which Ser-46 was replaced with other amino acids, suggested that ATP-dependent phosphorylation of HPr is involved in maltose exclusion in this organism (51). The participation of P-Ser-HPr in maltose exclusion has been confirmed by the results obtained with the *L. casei hprK208*(Am) mutant LcG102. Maltose uptake or consumption by the wild-type strain was prevented by the presence of glucose in the medium (Fig. 5A and 6A), whereas with the *hprK208*(Am) mutant addition of glucose led to increased maltose uptake, and maltose and glucose were found to be simultaneously consumed (Fig. 5B and 6B). Glucose uptake by the *hprK208*(Am) mutant was not altered when compared to the wild-type strain. It is therefore likely that P-Ser-HPr plays a role similar to unphosphorylated $EIIA^{Glc}$, the regulatory protein involved in inducer exclusion in *E. coli* (35), by inhibiting certain non-PTS transport systems when a rapidly metabolizable carbon source is taken up by gram-positive bacteria. However, our results could also be explained by assuming that the maltose permease is activated by P-His-HPr-catalyzed phosphorylation similar to the lactose permease of *Streptococcus thermophilus* (15) and that this activation is prevented when P-Ser-HPr is formed in response to the rapid uptake and metabolism of a carbohydrate.

The ATP-dependent phosphorylation of HPr at Ser-46 by HprK/P was discovered in connection with a regulatory phenomenon called inducer expulsion (7, 39). In several lactic acid bacteria, including lactobacilli (3), the addition of glucose to cells preloaded with a nonmetabolizable sugar derivative taken up by the PTS and therefore accumulated as phospho compound was found to cause the rapid expulsion of the sugar derivative in its dephosphorylated form (41, 46). Since the same conditions, which lead to inducer expulsion, were also found to trigger the in vivo phosphorylation of HPr at Ser-46, P-Ser-HPr was thought to play a role in inducer expulsion (39). However, as *ptsH1* or *hprK* mutants were only available for *B. subtilis*, and since no nonmetabolizable PTS sugars submitted to inducer expulsion are known for this organism, no in vivo experiments addressing the role of P-Ser-HPr in inducer expulsion have yet been carried out. We therefore studied inducer expulsion in *L. casei* and could show that this organism accumulates TMG as a phosphorylated derivative and that addition of glucose to cells preloaded with [^{14}C]TMG-6-P led to the expulsion of unphosphorylated TMG, similar to that observed in *Streptococcus pyogenes* (39). The Ser-46-Ala and Ser-46-Thr *ptsH* mutations, which caused relief from CCR (51), had no effect on glucose-triggered expulsion of preaccumulated [^{14}C]TMG-6-P (Fig. 7). Nevertheless, it was possible that, similar to *B. subtilis* (12), *L. casei* might possess an HPr-like protein, which could be operative in inducer expulsion, or the metabolite-activated HprK/P might phosphorylate an un-

known protein implicated in inducer expulsion. However, the *hprK208*(Am) mutant, which is devoid of HPr kinase activity, exhibited expulsion of preaccumulated [^{14}C]TMG-6-P identical to the wild-type strain, i.e., [^{14}C]TMG-6-P was dephosphorylated in the cells before unphosphorylated TMG was expelled. These results clearly established that HPr kinase activity is not required for TMG expulsion in *L. casei*. As the mechanism of TMG expulsion in *L. casei* strongly resembles the mechanism of inducer expulsion operative in streptococci and lactococci (41, 46), the results obtained in this study question the proposed implication of P-Ser-HPr in inducer expulsion of gram-positive bacteria.

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